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REVIEW

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Trace element speciation by ICP-MS in large biomolecules and its potential for proteomics

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Abstract Latest studies on the chemical association of trace elements to large biomolecules and their importance on the bioinorganic and clinical fields are examined. The complexity of the speciation of metal-biomolecules associations in various biological fluids is stressed. Analytical strategies to tackle speciation analysis and the-state-of-the-art of the instrumentation employed for this purpose are critically reviewed. Hyphenated techniques based on coupling chromatographic separation techniques with ICP-MS detection are now established as the most realistic and potent analytical tools available for real-life speciation analysis. Therefore, the status and potential of metal and semimetals elemental speciation in large biocompounds using ICP-MS detection is mainly focused here by reviewing reported metallo-complexes separations using size-exclusion (SEC), ion-exchange (IE), reverse phase chromatography (RP) and capillary electrophoresis (CE). Species of interest include coordination complexes of metals with larger proteins (e.g. in serum, breat milk, etc.) and metallothioneins (e.g. in cytosols from animals and plants) as well as selenoproteins (e.g. in nutritional supplements), DNA-cisplatin adducts and metal/semimetal binding to carbohydrates. An effort is made to assess the potential of present trace elements speciation knowledge and techniques for "heteroatom-tagged" (via ICP-MS) proteomics.

Keywords Trace element speciation · ICP-MS · Chromatography · Large biomolecules · Biological samples · Bio-inorganic proteomics

Speciation analysis evolution

The importance of "chemical speciation" and speciation analysis of trace metals [1] had been predicted more than 20 years ago by analytical electrochemists [2]. Along the years, however, this field has been continuously evolving, trying to cope with increasing demands for more complete information about the particular compound (chemical species) where the sought element is found in the analysed sample. Its importance is continuously growing, since the particular chemical form (species) present in the sample dictates the toxicity, transport and environmental impact of the considered metal or element.

It is known that the interest in speciation analysis was initially driven by environmental concerns: (antropogenic) organometallics of Pb, Hg or Sn had been released in the atmosphere, water and sediments and their toxicity posed a serious threat to exposed living organisms (and human health). Hybrid techniques in which a powerful separation of the sought species was coupled with an element-specific detector (mainly atomic detectors) were soon established as the most realistic and potent analytical tools available for real-life speciation analysis [3].

Natural developments and application of such "hybrid" techniques to obtain speciated information in environmental issues were soon after extended to the speciation of trace elements in biological material of the most varied living organisms. However, the complexity of chemical speciation in biological samples was greater than in environmental issues. In fact, for a given element many unknown compounds (e.g. unidentified chromatographic peaks containing that element) were detected as a result of its metabolism in the living organism under study. Particularly numerous were the elemental species detected using an ICP-MS as the on-line detector, due to ICP-MS extreme sensitivity, coupled to a high-performance liquid chromatographic column (HPLC). The mainly aqueous and polar nature of biological fluids explains why today the hybrid technique which holds the highest potential for element speciation in biological material is HPLC-ICP-MS [4, 5]. Of course, capillary electrophoresis (CE)-ICP-MS-coupled techniques also have to be considered for the purpose, although their present applications to real-life speciation problems are rather scarce [6].

As we will see in the next section, trace elements play very important roles in living organisms. In order to under-

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stand their essential, toxic or therapeutic functions it seems clear that their associations to key biomolecules (e.g. DNA, RNA, polypeptides, proteins, etc) should be established.

The ICP-MS is today the most powerful elemental detector to "screen" the presence of a given element in known or unknown large biomolecules, paving the way to further studies using "organic" MS techniques (ESI-MS, MALDI-TOF, ESI-$(MS)^n$) and also to biochemical methods to further investigate the nature of the biomolecule(s) bound to that element [7, 8]. Therefore, only work carried out on speciation analysis in large biomolecules using the ICP-MS (for the final specific detection of the sought element, that we will term "element-tagged" detection) will be reviewed here.

ICP-MS analytical potential for "element-tagged" proteomics

Many proteins and enzymes contain metals (or semi-metals) and it is well established that their biological activity will depend on the presence of the metal. Dioxygen transport (e.g. Fe^{3+} in oxy/deoxyhaemoglobin), electron transfer (e.g. Fe^{3+} in cytochromes), structural roles (e.g. Zn^{2+} "fingers"), metalloenzymes (hydrolytic, redox, methylation or rearrangement reactions) are good examples of such important roles of metals in proteins [9]. Of course, metal ion transport through membranes further illustrates how knowledge of metal–biomolecule associations is needed to understand very important neurological and triggering reactions. Studies into the transport and storage of metals are also needed to explain the differential accumulation of certain metals in "target" organs (e.g. brain and bones disorders specifically caused by Al^{3+} deposition [10] in such particular tissues). Nature has learned how to use ions as triggers for specific cellular functions and to regulate gene expression. Communication roles for metals in biology are nowadays well established: Na^+, K^+ and Ca^{2+} are commonly used to trigger important cellular responses (e.g. Ca^{2+}in calmodulin [11]). Finally, gene control by trace metals, as a form of biological signalling, has also been recognized via selective DNA binding to the adequate metalloproteins [8].

As ICP-MS is a specific detector for metals (or semi-metals) at extremely low levels, its synergic use with a previous high-resolution separation of the protein (or its complexes) offers a revolutionary analytical tool. Such hybrid instrumentation allows studies on metal–biomolecule associations/dissociations and, in doing so, on the mechanisms of biological action of metals (or semi-metals) in the organism. It should be stressed that the most versatile and used analytical techniques in proteomics are also "hybrid" techniques [12]. Mass spectrometry-based techniques play an increasingly major role in proteomics [13] as well as in speciation. Protein identification and determination after a high-resolution separation (e.g. 2D gel and capillary electrophoresis, orthogonal HPLC, etc.) of the corresponding biomolecule is commonly achieved by MS techniques.

In the following sections all the work carried out so far on metal and semi-metal elemental speciation in large biocompounds using ICP-MS detection will be revised. Of course, this approach can be further extended using alternative heteroatoms of the biomolecules to be investigated for instance, non-metals (P- or S-proteins) are starting to be investigated by ICP-MS and this is an area of great present interest because of their enormous importance to life and also the huge amount of biochemical knowledge already available.

Elemental speciation in large biomolecules by ICP-MS

Metals and metalloids can be found in nature as part of many biomacromolecules (as detailed in the preceding section). In living organisms the intake, accumulation, transport, storage and activity of a given element will be strongly influenced by its specific chemical form in the biological sample, that is to say, information on trace elements speciation in biological samples is today mandatory to understand the biochemistry of metals and semi-metals [4, 5, 8]. As mentioned in the previous section, most speciation studies have been approached through hybrid techniques monitoring the desired metal or semi-metal associated to the biomolecules [14]. It is important then to review the main reported bioligands which have been identified so far by resorting mainly to HPLC- or CE-ICP-MS. Three main groups of biomolecules can be distinguished: proteins, nucleic acids and carbohydrates.

Proteins and polypeptides

This is undoubtedly the group of metal–biomolecules more commonly investigated. Simple proteins are composed only of amino acids, while conjugated proteins are those that also include non-amino acid molecules in their structure. The non-amino acid group is called a *prosthetic group* and conjugated proteins can be classified (according to their prosthetic group) in lipoproteins (lipid), glycoproteins (carbohydrate), metalloproteins (metals, in many cases cofactors of enzymes), nucleoproteins (DNA, RNA) and phosphoproteins (phosphate). In general terms, proteins and conjugated proteins are bioligands which may form coordination complexes of different stability constant with essential (and with toxic) metals and semi-metals [7, 8]. Most of the metal–bioligand complexes described are associated with essential biological functions in living systems including transport of essential elements like Fe by human serum transferrin [15] or Cu by ceruloplasmin [16]. Catalytic roles (metallo-enzymes), like Zn in alcohol dehydrogenase and carbonic anhydrase [17], are of paramount importance. Similarly, essential elements associated to proteins have been studied in breast milk in an attempt to assess their nutritional values [18].

A common feature of this type of metal–protein biocompound is the well-known metal–bioligand association mechanism. As for other coordination complexes, the re-

lease of the metal from the bioligand can be described by the simple equation:

$$ML_n + nH^- \rightleftharpoons M^{m+} + nLH$$

and so the extent of the association is given by the corresponding conditional stability constant, K, in the biological environment (i.e. the pH value and all possible competitive ligands will condition the available "free" metal). Thus, the metallobiocompounds formed with proteins (and polypeptides) induced by heavy toxic metal exposure in plants and animals can also be classified in this main group of coordination complexes. The production of metallothioneins (MTs) in animals [19] or phytochelatins (that is, polypeptides) in plants [20] by exposure to well-known "toxic" elements (e.g. Cd, Hg or As) is widely documented in the literature. However, essential elements such as Cu^{2+} or Zn^{2+} are also strongly bound to MTs, which explains why both detoxification and homeostatic roles have been attributed to MTs [19]. From a chemical perspective, both MTs and phytochelatins are characterized by their high content on cysteine residues which provide their SH groups for eventual metal binding.

A second main group type of associations between a heteroatom and a protein is found in selenoproteins. Due to the non-metal character of Se (which mimics sulfur behaviour in biochemistry), this heteroatom can form part of the primary structure of the protein (i.e. Se can be incorporated into the biopolymer chain during expression of a given gene). In fact it is well documented that Se-cysteine amino acid is incorporated naturally in the main protein structure through the recognition in the UGA codon [21] (e.g. synthesis of glutathion peroxidase). Of course, in contrast with the coordination-type associations, the release of the semi-metal from the biopolymers usually requires a previous breaking down of the protein (e.g. enzymatic hydrolysis).

DNA/RNA fragments

Although to a much lesser extent than protein and polypeptides associations to metals and semi-metals, some work on speciation of large biomolecules by ICP-MS has also been reported, related to characterizing the DNA fragmentation induced by the action or certain chemicals [22] (e.g. polyaromatic hydrocarbons, PAHs). Such work suggested that ICP-MS could not only follow metals and semi-metals, but also P associated to large biomolecules could be monitored. Of course, in that way formation of P-containing oligonucleotides produced during DNA fragmentation processes caused by a specific chemical compound could be followed by a P-specific detector (e.g. ICP-MS). Similarly the mechanisms of interaction of some therapeutic drugs containing elements such as Pt or Au with DNA have been studied. In fact, anticancer activity of cisplatin (*cis*-diamminedichloroplatinum) is believed to result from its interaction with DNA. The drug reacts with nucleophilic sites in DNA forming mono-adducts as well as intrastrand and interstrand crosslinks. DNA–cisplatin adducts are specifically recognized by several proteins and some speciation work has been devoted to the interaction between cisplatin and plasma proteins [23].

Polysaccharides

Several reports have been published on As speciation in low molecular weight arsenic sugars. However, very few speciation studies have been carried out on compounds formed by polysaccharides with metals and semi-metals. In comparison with proteins, very little is known about the relevance of metal binding to carbohydrates, the most abundant compounds in the biosphere. In any case few examples are available [24, 25] and they will be illustrated in the following section of applications in plant material.

Selected applications

Body fluids

Serum and whole blood

It is important to note that most applications reported on the use of ICP-MS as specific detector for trace elements speciation in large molecules in living environments refer to human serum proteins. The majority of plasma proteins are glycoproteins, albumin being an exception [26]. For

Table 1 Major constituent proteins in human plasma in the five electrophoretic fractions (separated at pH 8.6)[a]

Fraction	Relative percent (%)
Albumin	52–68
Alpha-1	2.4–4.4
Thyroxine-binding globulin	
Transcortin	
Alpha-1-acid glycoprotein	
Alpha-1-antitrypsin	
Lipoprotein	
Alpha-2	6.1–10.1
Haptoglobin	
Macroglobulin	
Ceruloplasmin	
Beta	8.5–14.5
Transferrin	
Hemopexin	
Lipoprotein	
Gamma	10–21
IgG	
IgM	
IgA	
IgD	
IgE	
C-Reactive protein	

[a]Adapted from ref. [26]

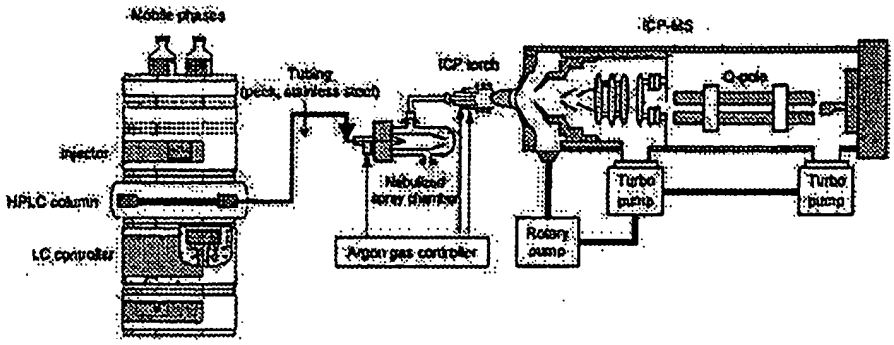

Fig. 1 Schematic of the coupling of liquid chromatography to inductively coupled plasma mass spectrometry (HPLC-ICP-MS). Diagram kindly provided by Agilent Technologies

comparison, the major constituent proteins (relative percentages) in human serum are summarized in Table 1. Serum, usually obtained from whole blood by centrifugation, is an extremely complex matrix that is normally just diluted in aqueous buffers for speciation purposes.

Speciation analysis of human serum proteins has mainly been carried out by HPLC-ICP-MS so far. This coupling, illustrated in Fig. 1, is straightforward: the exit of the column is connected to the entrance of the ICP nebulizer; the main precaution to be taken is to ensure the compatibility of the mobile phases and the ICP-MS running. The preferred chromatographic mechanisms have been anion exchange (AE) and size exclusion (SEC), in order to minimize any changes in the existing species (both techniques allow the use of biological buffers and physiological pH in the mobile phases) [5, 14, 27]. Separations by size exclusion chromatography (SEC), based on the molecular size of the species, can be used as a first approximation to the molecular weight distribution of the species present in the biological sample. Complementary separations providing matching results [4] are often needed for a separation with the required resolution. For instance, it is known that SEC is not capable of resolving albumin (66 KDa) from transferrin (80 KDa) and so AE further separations, after SEC, are commonplace to solve such problems. When low molecular compounds are of interest, ultrafiltration through a 10-KDa membrane is normally used before the chromatography is carried out on the ultrafiltrate.

On the other hand, AE chromatography is mainly dependent on the ionic nature of the species to be separated and, in the case of proteins, on their isoelectric points. The main limitation of using AE arises from the requirement of drastic elution conditions (high salt concentration) in order to elute some of the species in a reasonable time as this can compromise the ICP-MS's long-term performance (due to deposits on the cones, etc.). In this sense, ammonium acetate buffers have been preferred to perform anion exchange chromatography coupled to ICP-MS [14].

Figure 2 shows the separation of essential elements (Fe, Cu and Zn) in human serum proteins with a gradient of ammonium acetate using AE chromatography and double-focusing ICP-MS detection [28]. These three elements have been extensively studied in relation to human serum proteins. The main storage protein of Fe is ferritin, mostly found in tissues. When Fe is required by the body, it is released by ferritin into the plasma where is transported by transferrin [26] (see Fig. 2). Cu is also transported by ceruloplasmin in human plasma (about 90% of the total Cu) and also is associated to albumin [29, 30]. Finally Zn is a constituent in more than 200 enzymes and in human plasma can be found in association to α_2-macroglobulin and albumin [16].

However, some other elements considered "toxic" have been also found in human serum. Thus, long-term uraemic patients treated by haemodialysis exhibit elevated concentrations of elements such as Al, Cr or Pb in plasma and Al and Cr are associated to transferrin fractions [31]. This protein binds about 90% of the total Al present in human serum [32, 33] and the remaining 10% has been found in low molecular weight fractions (<10 KDa) and characterized by ES-MS as Al–citrate complexes [34]. Cr and Pb have been also explored in haemodialysis patients and workers exposed respectively. Cr has been identified in transferrin fractions while Pb has been mainly associated to ceruloplasmin [35, 36] and to a lesser extent, to an unidentified protein of about 600 KDa. In the erythrocytes of exposed workers, Pb appeared to be bound to a main species of about 240 KDa [35].

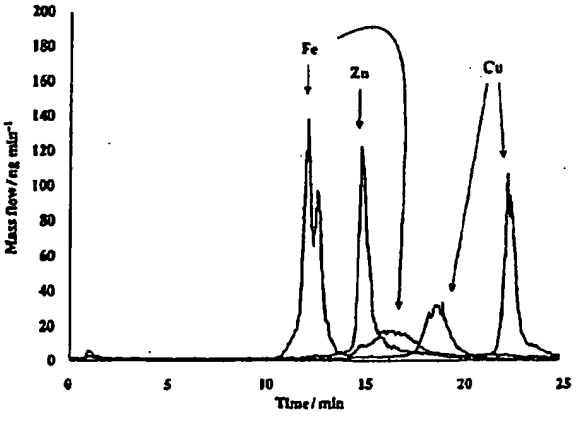

Fig. 2 Chromatogram of the elements associated to human serum proteins (Fe, Cu and Zn) separated by anion exchange chromatography and detected by double-focusing ICP-MS (resolving power 3,000). The mobile phase consisted on an ammonium acetate gradient at physiological pH. Reproduced from Sariego Múñiz C (2001) J Anal At Spectrom 16:587, with permission

Affinity chromatography is also a widely used separation mechanism for the isolation of proteins in biological fluids. In this mode, the separation is based on specific interactions of some proteins with certain molecules (biospecific ligands) which are immobilized into an Agarose solid phase. Since the interactions with the stationary phase are protein-specific, this separation mechanism is more commonly used as purification process of a single protein from a mixture and no applications in the literature of the on-line coupling of such separation to ICP-MS detection has been found yet. However, this has been the preferred separation mechanism used for the isolation of certain Se-proteins. The purification of Se-protein P in human serum has been accomplished by metal-ion affinity chromatography and off-line ICP-MS detection [37]. The fraction containing the Se-protein P has been further characterized by polyacrylamide gel electrophoresis (PAGE) obtaining two isoforms of the protein. Glutathion peroxidase and selenoprotein P have been found in rat serum by means of SEC-ICP-MS [38]. However, recent literature has described the presence of more than 10 Se-containing proteins whose functional characterization has not yet been discovered [21].

Finally, some studies have also illustrated the association of precious or semi-precious metals complexes with human serum proteins due to the extensive use of these complexes as anti-tumoural drugs. Cisplatin, carboplatin and some other Ru complexes have been employed to treat different tumours. However, the activity of those complexes can be extremely different when associated to a certain protein. The experiments performed by SEC-ICP-MS, incubating the cisplatin drugs with serum, showed evidence of association of the drug to a serum molecular fraction of 60 KDa [23].

Milk

Fractionation techniques of metals in milk are now needed. The distribution of essential and toxic elements among the different fractions of milk (fat, caseins and serum) is a previous step to the study the particular chemical forms in which they are present in each fraction. Multi-elemental distribution patterns from human, cow and formula milks have been compared [39]. Total elemental distributions in skimmed milk and/or whey milk samples are obtained after removing milk fat and/or caseins by ultracentifugation. Results in whole and skimmed milk showed also that essential elements (including, Mg, Mn, Fe, Zn, Cu and Se) could also in part (about 25%) be associated to the fatty fraction.

The elemental distribution observed in human milk was significantly different to that observed in cow or formula milks [39]. After separation of caseins, less than 40% of Ca, Mn, Fe, Se, Zn and Cu were present in serum of cow and formula milk samples. Toxic elements (i.e. Al, Pb, Cd) in cow and/or formula milk samples were associated to caseins. Essential elements found in human milk serum amounted to about 70% of the total in milk, probably due to the comparatively lower level of caseins present in human milk than in the other milk types. The next step, the identification of the possible chemical species in which an element is found in each milk fraction has also been undertaken. The largest interest so far is focused on trace element speciation of Fe, Cu, Zn and I and also toxic elements (mainly Al, Pb and Cd) distribution and speciation. Usually SEC separation techniques coupled to ICP-MS detection has been used [18, 40, 41, 42].

In order to minimize interactions between the column and the labile metal bound to the bio-organic compound, a size exclusion column (SEC) is preferred in milk whey. Copper in human milk and raw cow milk appears to spread over many bio-compounds from high to low molecular weights [18]. The main two peaks seem to appear one in the high molecular region (>180 kDa), associated to immunoglobulins and caseins residues, and other at 94 kDa, in the serum albumin and lactoferrin region. In formula and UHT milk whey some Cu peaks disappeared, probably due to a denaturalisation of those proteins during the elaboration process. In the case of Fe, Rivero et al. [18] and Coni et al. [40] have described the association of Fe to both large proteins (>180 kDa) and to low mass compounds (<1.4 kDa) in breast milk. However, Bratter [41] postulated that in breast milk Fe is mainly bound to lactoferrin. In milk formulas the binding patterns turned out to be very different.

Also for Zn, the distribution observed in human milk strongly differs from cow and formula milk. In human milk, zinc is spread in the fractions corresponding to lactoalbumin, caseins and immunoglobulins, albumin, lactoferrin and also citrates [18, 40, 43]. Conversely, in cow and formula milks, Zn was found in the high molecular fraction region (caseins and immunoglobulins) and citrates.

Selenium speciation by SEC in skimmed human samples has been carried out using on-line detection by ICP-MS with a hexapole collision cell and just one peak was observed at 6 kDa [42]. The distribution patterns of selenium species in cow, breast and formula milk have been also compared [41] and a minimum of five selenocompounds were detected in breast milk (mass range 10–300 kDa). The soy-based formulas analysed showed different speciation profile than cow-based formula milk for selenium. Moreover, selenium peaks observed in cow and soy formulas occurred at retention times quite different to those observed for human breast milk.

In the case of Ca, a small fraction of the total calcium is bound to bioligands of the medium molecular region (39 kDa) [43], while in cow milk and formula milk Ca is mainly present in casein micelles.

Iodine in milk whey was present as iodide and as traces of unidentified organic iodine compounds (>100 kDa) [44, 45].

The natural distribution of S, P and Br in human skimmed milk sample has also been reported [42]. There is some S detected in larger proteins (>150 kDa) but it mostly occurs at 36 kDa, while P and Br appeared associated to low molecular weight proteins.

It is interesting to note that toxic elements in milk whey seem to be associated to high molecular mass compounds

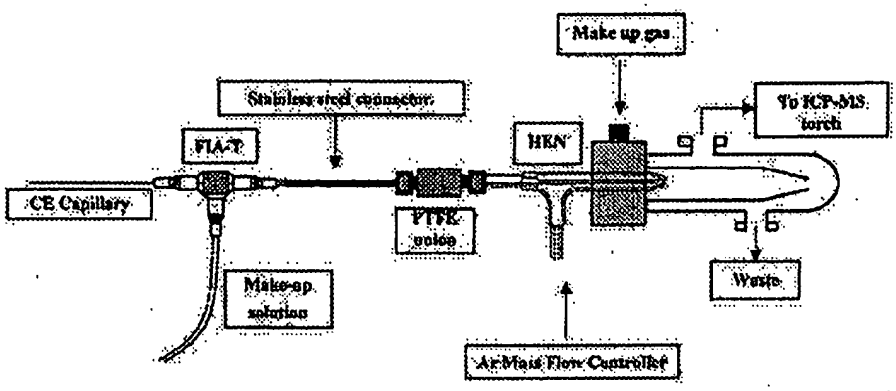

Fig. 3 Schematic of an "in-house" interface to couple capillary electrophoresis to ICP-MS using the high efficiency nebulizer (HEN)

(e.g. caseins and immunoglobulins), being absent in the non-proteic fractions [40].

Urine and amniotic fluid

It is well known that urine is a complex matrix with high saline concentration, containing also low molecular mass organic molecules (below 15 kDa). Metals monitoring in urine provides information on the balance of essential elements and on possible exposure to toxic ones (e.g. As, Cd, Hg, Pb). In addition, the chemical form in which they are present is of great importance to elucidate its metabolism and detoxification processes. Although most research performed on metal speciation in urine concerns small molecules, metal speciation of metallothioneins (MTs), has also been reported for Cd in urine by Sanz-Medel's group using "vesicular" HPLC-ICP-MS [46]. The main cadmium species found in basal urine seem to be similar to rabbit liver MT-1.

Human fetus grows up swallowing and inhaling a urine-like fluid called amniotic fluid. This fluid is absorbed by gastrointestinal tract and transferred to fetal blood. Since it was established that Pb can cross the placenta and was detected in fetus tissues and amniotic fluid, it is important to know the bioavailability of the Pb in amniotic fluid. Hall et al. [47] showed that Pb was mainly bound to a Zn-peptide (5 kDa) with antibacterial activity and ceruloplasmin.

Animal tissues

Probably the more investigated bioligands for toxic metal ions in vertebrate animal tissues are metallothioneins (MTs). Also, it has been suggested that the concentration of so-called metallothionein-like proteins (MLPs) in marine invertebrates might serve as a biomarker of metal pollutants in aquatic environment [19]. Speciation analysis of MTs and MLPs by HPLC coupled to ICP-MS seems to be adequate for detecting metal–biomolecule complexes in cytosols from animal tissues [48, 49, 50, 51, 52, 53]. The majority of such studies concern the speciation of Cd, Zn and Cu in kidney [54, 55, 56], liver [46, 49, 57, 58, 59, 60, 61, 62, 63, 64] and brain [65] of mammals (rat, rabbit, bovine, humans) or tissues of bream [66], carps [67], eels [68] and mussels [69, 70, 71]. MT isoforms have been separated by size SEC, anionic exchange-HPLC and reverse phase (RP)-HPLC chromatography.

Capillary electrophoresis has great potential for the separation of metallothionein isoforms and sub-isoforms, differing only in a few amino acids, and speciation using CE-ICP-MS as been published by monitoring S and Cd, Zn, Cu in MTs [6, 48, 60, 72]. The major problem in coupling CE to ICP-MS is the interface design. A number of interfaces with concentric modified nebuliser [73], ultrasonic nebuliser [74], high efficiency nebuliser [75], microconcentric [75, 76, 77] and cross flow nebuliser [78] have been applied to the speciation of metals bound to MTs. A typical interface used in our group, CE-ICP-MS, is illustrated in Fig. 3, while Fig. 4 shows some typical electrophoretic results using such a hybrid technique for speciation of Cd metallothioneins [75].

Advantages and limitations of quadrupole (Q), double-focusing (DF) and time-of-flight (TOF) mass analysers

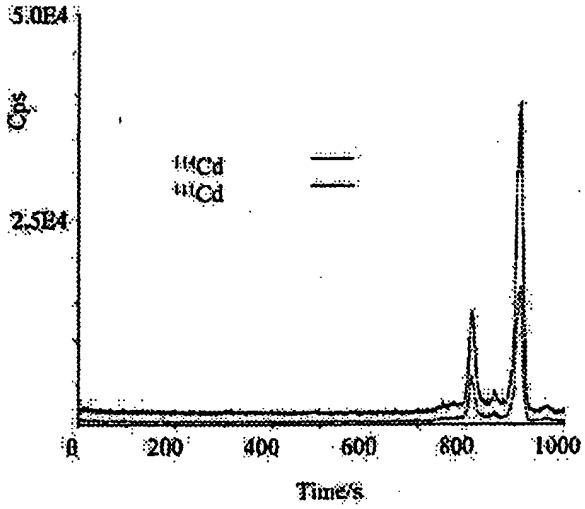

Fig. 4 Separation of metallothioneins (isoforms 1 and 2) by CE-ICP-MS. Alvarez Llamas G et al. (2002) J Anal At Spectrom 17:655, with permission

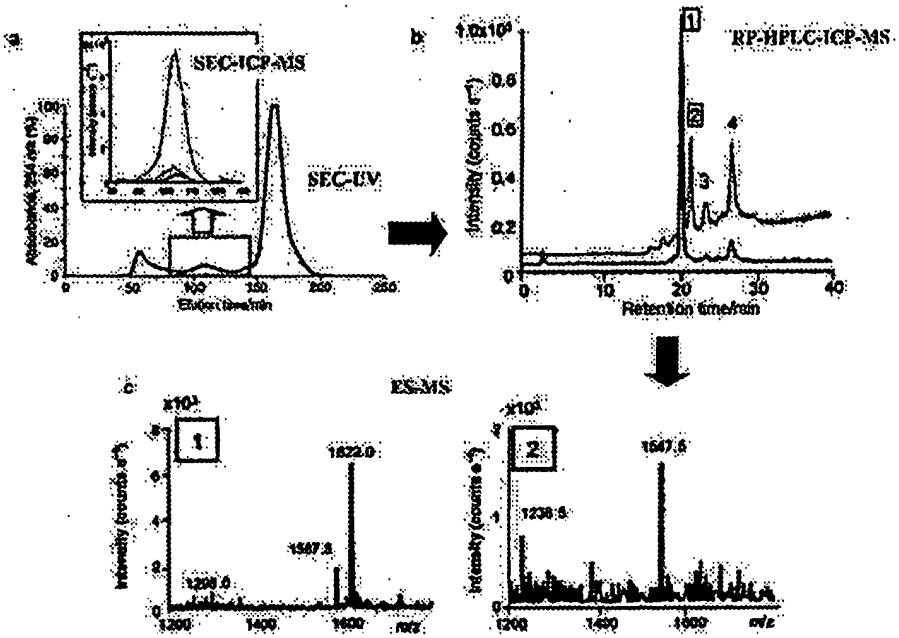

Fig. 5a–c New trends in speciation: separation of Cd–metallothionein isoforms by a SEC with UV and ICP-MS detection, b reversed-phase ICP-MS of the main Cd-containing species eluted by SEC, c ES-MS of the peak labelled as *1* and *2* in the RP-HPLC-ICP-MS chromatogram. Reproduced from reference Polec K et al. (2000) J Anal At Spectrom 15:1363, with permission

for multi-elemental speciation of metallothioneins have been reported [49]. Polyatomic interferences could be avoided by working at medium resolving power in a (DF) ICP-MS and this instrument was proposed for accurate MT quantifications by isotope dilution analysis [64, 79]. A stable isotope ratio measurement ($^{114}Cd/^{111}Cd$) has also been proposed for the determination of oxidized MTs in biological samples [80].

Figure 5 illustrates with a typical example the new trends in speciation analysis to identify/confirm the bioligands associated to the heteroelement of interest. The initial separation by SEC with UV and ICP-MS detection of Cd-metallothioneins in rat liver is shown there. Figure 5b shows that Cd-containing species found are further separated and controlled by RP-HPLC-ICP-MS. Finally, the most intense chromatographic peaks (labelled as 1 and 2 in the RP chromatogram) are eventually identified by ES-MS.

Plant material

Studies on plant material, in terms of the presence and associations of trace elements with large biomolecules, are mainly related to phytoremediation processes (a technology to clean-up polluted sites from contaminants such as Se, Cd or Pb). Two major heavy-metal binding compounds are known in plant cells: phytochelatins (peptides, PCs) and metallothioneins (proteins, MTs). Both bioligands are cysteine-based metal ligand systems, but with a different biochemical origin and very different molecular weights: PCs have molecular weights in the range 200–3,000 Da and MTs are about 6–8 KDa [20]. Very few studies have been carried out on the characterization of PC or MT associations to metals using ICP-MS detection. The scarce published work in this field used SEC as separation mechanism and ICP-MS for the detection of the element associated to the corresponding PC [81, 82]. The analysis of plant extracts (extracted using Tris-HCl at pH 8) of *Silene cucubalus* and *Agrostis tenuis* revealed the presence of multiple complexes, from PC_2 to PC_4 and also the presence of higher molecular weight species. Further characterization of the involved peptides has been attempted by electrospray MS [83]. Recent studies have been also carried out on the speciation of Ni in the latex of a hyperaccumulating tree (*Serbetia acuminata*) by HPLC and CZE with ICP-MS detection [84]. The evaluation of the extracts by ES-MS showed the presence of a Ni–nicotianamine complex (0.3% of the Ni extracted) and mainly a Ni–citrate complex (99.4% of the Ni).

Foodstuff of plant origin contains also high concentrations of polysaccharides of which the potentially negatively charged oxygen functions can bind cations. In comparison to proteins, very little is known about these compounds and some studies have been performed in order to characterize them in fresh fruits and vegetables [24]. Most of this work has been done by means of SEC as separation mechanism to estimate the molecular weight of the element–biomolecule association. The study concluded that the Pb, Ba, Sr, Ce and B were associated to the high molecular polysaccharides fraction (>50 kDa), whereas Zn, Cu and Mg eluted with low molecular mass non-carbohydrate compounds [24].

Nutritional supplements and food

The vast majority of studies in the area of elemental speciation in nutritional samples have been devoted to sele-

nium speciation [85]. The knowledge of the nature of the selenocompounds produced by yeast grown in a selenized nutritive medium is paramount in terms of its nutritional value. A critical step in such speciation analysis is the extraction of trace selenocompounds from the solid. A soft extraction with water or methanol led only to 10–20% selenium recoveries, while extraction with or sodium dodecyl sulfate solution or proteolytic enzymes led to recoveries of 40 and 80%, respectively. Size exclusion chromatography was used to separate selenium species present in the different yeast extracts and showed that about 75% of yeast selenium was bound to high molecular mass compounds (proteins, nucleic acids or cell walls) or present in water-soluble proteins [86].

Fish accumulate important amounts of selenium and constitute an important dietary source of element. Size exclusion chromatography coupled to ICP-MS was used to study the soluble selenocompounds in fish, showing large differences among selenocompounds present in different fish species [87].

Nuts could be used as Se supplements because high levels of Se have been found in nuts. The elemental distribution among different fractions (liquid extract, low molecular weight and protein fraction) and Se speciation has been reported in different types of nuts [88].

Finally, fractionation of soluble species of P, Mn, Fe, Co, Ni, Cu, Zn, Se and Mo in pea and lentil seeds with SEC-ICP-MS was reported recently [89]. Chromatographic profiles of both matrices turned out to be similar for all elements, except Mo. Most relevant applications of ICP-MS as detector in large biomolecules speciation as summarised in Table 2.

A forward look into "heteroatom-tagged" speciation analysis

A natural trend of the present popular use of ICP-MS to investigate speciation in metal-containing biomolecules is to extend its application to "screening" certain semi-metals or even for the presence of non-metals in those molecules. The ability of ICP-MS to monitor a given isotope (and element) with great sensitivity and specificity could be advantageously exploited in studies of biomolecules containing S, P, Br or Si heteroatoms.

There are already some illustrative examples of the application of S-tagged ICP-MS speciation for volatile species with GC [90, 91] and for larger molecules using HPLC pre-separation [69]. Phosphorous has been proposed as the tagged element for quantitative determination of DNA adducts by HPLC-(HR) ICP-MS [92] and for the determination of phosphoprotein phosphorylation degrees by capillary liquid chromatography-ICP-MS [93, 94]. The same authors have reported recently the use of the above technique in a typical proteomics search to identify phosphorylation sites in kinase-type proteins [95].

Interfacing laser ablation with ICP-MS for the direct analysis of proteins separated in electrophoresis gels has also been proposed using Co-tagged speciation [96]. Similarly P-tagged laser-ICP-MS detection was used to ascertain the presence of phosphorylated proteins on electrophoresis gel blots [97]. This laser-based technique has proved to be useful for selenoproteins speciation as well [98]. Even continuation of some pending studies of Si binding to proteins [99] could be now envisaged by Si-tagged HPLC-ICP-MS in a similar manner to that described by GC-ICP-MS [100].

In short, specific heteroatom-tagged investigations can now provide great advantages in proteomics (it has been proved by now for metal/semi-metal-biomolecules), as they can give complementary information to that obtained so far using common molecule-selective detectors (e.g. molecular absorption, fluorescence, IR, NMR, MS, etc).

In particular, proteomics research via hybrid techniques with ICP-MS on-line detection could benefit from important ICP-MS features, including:

1. Extreme sensitivity. New "unknown" proteins, or their translational modifications, present at extremely low levels could be unveiled by ICP-MS multi-elemental detection in the separated biocompounds. This would lead to further characterisation studies (e.g. by applying "molecular" MS techniques, such as MALDI-TOF, ESI $(MS)^n$,etc.) of the detected compounds [7].
2. Easy coupling to classical biochemical separations. Immunotechniques [101] and other powerful and popular biochemical planar separations such as PAGE can be coupled to ICP-MS for final "heteroatom-tagged" detection via laser ablation [96, 97].
3. Multi-elemental capabilities. Synergistic biological effects by several elements, for example, Hg and Se in selenoprotein P [102] or Ag, Cd, Hg and Se in selenoprotein P [103] or the effect of Se in Cd-metallothioneins speciation [104] could be easily investigated by studying their multi-elemental speciation simultaneously. ICP-MS capabilities are often spoiled in present speciation work and multi-elemental detection should be encouraged whenever possible for biological issues.
4. Isotopic detection. Many metabolism and nutrition studies could be conducted in vivo in human beings, as present radioisotopes in vitro experiments could be safely extended using enriched stable isotopes. Perhaps classic radiometric biochemical techniques (e.g. with ^{32}P) widely used to study P-mediated biochemical reactions such as post-translational phosphorylations [101] could be now complemented/replaced by non-radioactive stable isotope ICP-MS alternatives.

Exploitation of heteroatom-tagged speciation analysis for proteomics could also offer great advantages from a quantitative stand-point. At least two useful aspects can be foreseen: first, the direct use of available inorganic elemental standards for protein determination at extremely low concentrations in a very fast, on-line manner (hybrid technique) [100]; secondly, more accurate quantifications of such biocompounds (again at very low levels) can be attempted by adequate exploitation of speciated isotope dilution analysis (SIDA) [105, 106, 107], recently extended to multi-labelled multi-species SIDA techniques and demon-

Table 2 Most relevant applications on the use of ICP-MS as detector in large biomolecules speciation

Sample	Element	Separation mechanism	Buffer	Ref.
Biological fluids				
Serum	Cu, Zn	SEC: Superose 12HR	0.1 M Tris-HCl buffer+2.5 $CaCl_2$ (pH 7.4)	[16]
Serum	Fe, Cu, Zn	AE: Mono-Q HR	Ammonium acetate gradient (0–250) in 50 mM Tris-HCl (pH 7.4)	[28]
Serum	Fe, Cu, Zn, Mo, Se	ODS chemically coated with CHAPS	0.2 mM Tris-HCl+0.2 mM CHAPS (pH 7.4)	[30]
Serum	Fe, Cu, Zn, Ca, Cr, Mn, Pb, Se, Sr.	AE: Mono-Q HR	Ammonium acetate gradient (0–250) in 50 mM Tris-HCl (pH 7.4)	[31]
Serum	Al	AE: Mono-Q HR	Ammonium acetate gradient (0–250) in 50 mM Tris-HCl (pH 7.4)	[33]
Serum and erythrocytes	Pb, Fe, Cu, Zn, Mg	SEC: TSK G3000 SW	100 mM Tris-HCl (pH 7.2)	[35]
Serum	Cd, Cu, Fe, Pb, Zn	SEC: Synchropak GPC 300	100 mM Tris-HCl (pH 6.9)	[36]
Serum	Se	Off-line heparin sepharose-Co	1 M ammonium formate (pH 4)	[37]
Rat serum	Se	SEC: GS 520	50 mM Tris-HCl (pH 7.4)	[38]
Serum	Pt and Ru drugs	SEC: Progel TSK G3000 AE: TSK-gel DEAE	30 mM Tris-HCl (pH 7.2) Acetic acid gradient (0–500 mM) in 10 mM Tris-HCl (pH 7.4)	[23]
Human cow and formula milk	Fe, Cu, Zn, Mn, Sr, I, Br, Ca, Mg	SEC: TSK G 2000SW_{xl}	0.1 M Tris-HCl (pH 7.0)	[18]
Human milk	Ba, Bi, Cd, Cu, Li, Mg, Mn, Mo, Pb, Sr, Tl, Zn	SEC: TSK G 3000SW_{xl}	0.10 M HEPES 0.08 M NaCl (pH 6.8)	[40]
Human breast milk, infant formula	Fe, Mn, Cu, Co, Zn, Se, Mo, Cd, Pb I, Br, P, S	SEC: Shodex Asahipak GS 6520 HQ Shodex Asahipak GS 520 HQ	0.1 M Tris-HCl (pH 7.1)	[41]
Human milk	Mg, Ca, Cu, Fe, Mn, Ni, Zn, Cr, Se, Mo, S, P, Br	SEC: TSK G super 2000	50 mM Tris-HCl 0.05% NaN_3(pH 7.0)	[42]
Human milk	Al, Ba, Ca, Cd, Co, Cr, Cs, Cu, Fe, Li, Mg, Mn, Mo, Ni, Pb, Sr, Tl, V, Zn	SEC: TSK G 3000SW_{xl} TSK G 4000SW_{xl}	0.10 M HEPES 0.08 M NaCl (pH 7.0)	[43]
UHT and fresh milk	I	AE: DIONEX AS-14	3.5 mM Na_2CO_3 1.0 mM $NaHCO_3$	[44]
Milk and infant formulas	I	SEC: Superdex-75 Superdex-200	30 mM Tris-HCl (pH 7.0)	[45]
Urine	Cd	AE: DEAE-5PW	A: 2 mM Tris-HCL (pH 7.4), B: 200 mM Tris-HCL (pH 7.4)	[46]
Amniotic fluid	Pb, Cu, Zn	SEC: TSK G 3000SW_{xl}	100 mM Tris-HCl 0.1% propanol	[47]
Animal tissues				
Rat liver, kidney	Cd, Cu, Zn	RP: Microbore Vydac C_4 CB: 100 cm	A: 5 mM acetate in water (pH 6), B: 5 mM acetate 50% acetonitrile (pH 6) 12 mM Tris-HCl (pH 7.5)	[50]
Rat liver, kidney	Fe, Cu, Zn, Se	SEC: Asahipak GS 520	50 mM Tris-HCl (pH 7.5)	[54, 55]
Pig kidney	Cd	SEC: Pharmacia Superose-12	0.12 mM Tris-HCL (pH 7.2)	[56]
Rabbit liver	Cd, Cu, Zn	AE: DEAE 5PW	A: 2 mM Tris-HCl (pH 7.4), B: 200 mM Tris-HCl (pH 7.4)	[46]
Mouse liver	Cu, Zn, Se, Fe	SEC: Asahipak GS 520	50 nM Tris-HCl (pH 7.4)	[57]
Rabbit liver	Cd, Zn	RP: Microbore Vydac C_4	A: 5 mM acetate (pH 6), B: 5 mM acetate 50% methanol (pH 6)	[58]
Rabbit liver	Cd	RP: Microbore Vydac C_4	A: 5 mM acetate (pH 6), B: 5 mM acetate 50% acetonitrile	[59]
Rat liver	Cd, Cu, Zn	SEC: Superdex 75 RP: Vydac C_4	30 mM Tris-HCl 20 mM mercaptoethanol A: 5 mM acetate (pH 6), B: 5 mM acetate 50% acetonitrile pH 6	[60]

Table 2 (continued)

Sample	Element	Separation mechanism	Buffer	Ref.
Rat liver	Cd, Zn	SEC: TSK G 3000SW	25 mM Tris-HCl (pH 8.0)	[61]
		AE: Shodex Asahipak ES-502 N 7C	A: 2 mM Tris-HCl (pH 7.2), B: 50 mM Tris-HCl (pH 7.2)	
Bovine liver	Cu, Zn, Mn, Fe, Cd, S, P, Mo, Co, Ca, Mg	SEC: TSK gel G 3000SW$_{XL}$	50 mM Tris-HCl 0.05% NaN_3(pH 7.3)	[62]
Rabbit liver	Cd	AE: DEAE 5PW	A: 2 mM Tris-HCl (pH 7.4), B: 200 mM Tris-HCl (pH 7.4)	[63]
		Momo Q HR 5/5	A: 4 mM Tris-HCl (pH 7.4), B: 250 mM Tris-HCl (pH 7.4)	
Rabbit liver	Cd	RP: Vydac C_4	A: 2 mM Tris-HCl (pH 7.4), B: 2 mM Tris-HCl (pH 7.4), 50% methanol	[64]
Human brain	Cu, Zn, Cd, Pb, Ag	SEC: Superdex 75 PG	20 mM Tris-HCl (pH 7.4)	[65]
Bream brain	Cu, Cd, Zn	RP: Purosphere C_{18}	A: 30 mM Tris-HCl (pH 7.0), B: 30 mM Tris-HCl (pH 7.0) in acetonitrile	[66]
Carp liver, kidney	Cu, Cd, Zn	SEC: TSK gel G 3000SWXL	30 mM Tris-HCl (pH 7.4)	[67]
		AE: DEAE 5PW	A: 2 mM Tris-HCl (pH 7.4), B: 200 mM Tris-HCl (pH 7.4)	
Eel liver	Cd	"vesicular": C_{18} modified by BDDA	A: 2 mM Tris-HCl (pH 7.4) 1 mM DDAB, B: 200 mM Tris-HCl (pH 7.4) 1 mM DDAB	[68]
Mussels	Cd, Zn, Cu	AE: Momo Q HR 5/5	A: 4 mM Tris-HCl (pH 7.4), B: 250 mM Tris-HCl (pH 7.4)	[69]
Mussels	Cu, Zn, Ca, U, Ni, Mo, Mn, Cr, V, Cd, Al, Sb, Fe, Sn, Pb, Co Hg, Ag	SEC: Sephadex G-75	10 mM Tris-HCl 5 mM, 2-mercaptoethanol 0.1 mM phenylmethylsulfonyl fluoride 25 mM NaCl (pH 7.4)	[70]
Mussels	Al, Cu, Cd, Zn, V, Cr, Sn, U, Ag, HgPb, Sb, Co, Mn, Mo, Ni	SEC: Sephadex G-75	10 mM Tris-HCl 5 mM, 2-mercaptoethanol 0.1 mM phenylmethylsulfonyl fluoride 25 mM NaCl (pH 7.4)	[71]
		AE: Momo Q HR 5/5	A: 4 mM Tris-HCl (pH 7.4), B: 250 mM ammonium acetate 4 mM Tris-HCl (pH 7.4)	
Rabbit liver	Cd, Cu, Zn	CE 150 cm, 75-μm ID	20 mM Tris-HCl (pH 7.1)	[73]
Rabbit liver	Cd, Cu, Zn	CE 12 cm, 75-μm ID	20 mM Tris-HCl (pH 7.8)	[74]
Rabbit liver	Cd, Zn	CE 61 cm, 75-μm ID	20 mM Tris-HCl (pH 7.4) 5% methanol	[75]
Rabbit liver	Cd, Cu, Zn	CE 77 cm 50-μm ID	20 mM Tris-HCl (pH 7.8)	[76]
Rabbit liver	Cd, Zn	CE 110 cm 50-μm ID	50 mM Tris-HCl (pH 9.1)	[77]
Horse kidney, rabbit liver	Cd, Cd, Zn	CE 100 cm 50-μm ID	50 mM Tris-HCl (pH 9.0)	[78]
Rat tissue	Cd, Cu, Zn, S	CE 70 cm 75-μm ID	20 mM Tris-HCl (pH 7.4)	[79]
Plant material				
Plants	Cd	SEC: Superdex peptide HR 10/30	30 mM Tris-HCl (pH 8.5)	[81]
Plants	Cd, Cu, Zn	SEC: Eurogel GFC	10 mM ammonium acetate (pH 7)	[82]
Tree	Ni	SEC: Superdex Peptide HR 10/30	5 mM ammonium acetate (pH 6.8)	[83]
		CE 70 cm, 75-μm ID	5 mM ammonium acetate (pH 6.8)	
Nutritional supplements and food				
Yeast	Se	SEC: Superdex 200 HR10/30,	30 mM Tris-HCl pH 7.0	[86]
		AE: Hamilton PRP-X 100,	A: 6.25 mM ammonium phosphate pH 5.5, B: 25 mM ammonium phosphate pH 11	
		RP: ODS-2	0.1% TFA 2% methanol	
Fish	Se	SEC: Superdex 200 HR 10/30	20 mM Tris acetate 0.15 mM ammonium acetate pH 7.5	[87]
Nuts	Se	Superdex HR 10/30	50 mM ammonium acetate pH 4.5, 0.02% SDS	[88]
Seeds of legumes	P, Mn, Fe, Co, Ni, Cu, Zn, Se, Mo	Superdex 75 HR 10/30 Superdex Peptide HR 10/30	20 mM Tris-HCl pH 7.5	[89]

strated for sediments and biological tissues in our laboratory [108, 109].

In brief, so far ICP-MS has proved to be an invaluable tool for the "screening" of the presence of metals in biomolecules even at extremely low concentrations. However, such new metal (or "element-tagged") biopolymers, unveiled by ICP-MS, should then be identified and confirmed by "molecular" MS techniques [5, 7]. Integration of "atomic" and "molecular" MS studies, for example using HPLC-MS (TOF) and HPLC-ICP-MS on-line via flow splitting [110], will grow in the future.

Moreover, in order to unveil and understand the biological roles of metal compounds, typical biochemical techniques and methods should be applied from now on. In this vein, gene cloning, protein expression and purification with final characterisation of the expressed biocompound should be aimed at [7]. Cross-fertilisation and cooperation with organic chemists, biochemists and biologists seems unavoidable at this stage.

This "holistic" approach seems to be in the horizon of present chemical speciation which will soon become biochemical speciation (in order to be able to elucidate the role of metals in health and disease). Notwithstanding that trend, however, the very first step triggering such holistic research process is today to be able to detect new metal–biomolecules, likely using ICP-MS "on-line" detection. Moreover, "heteroatom-tagged" ICP-MS, as a favourable "marker" of certain biomolecules, will compete or complement other biochemical techniques and so play a key role in future proteomics research.

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